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EXPERIMENTAL STUDY

Evaluation of erythrocyte deformability in experimentally induced osteoporosis in female rats and the effects of vitamin C supplementation on erythrocyte deformability

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Abstract: *Objective:* The aim of this study was to evaluate the possible variations in antioxidant enzymes, lipid peroxidation and erythrocyte deformability in experimentally induced osteoporosis in female rats and to assess the effects of vitamin C supplementation on those variations.
Material: A total of 20 female Wistar Albino Rats were randomized into the three groups as controls (Group C, n=6), ovariectomized rats (Group O, n=7) and ovariectomized rats receiving vitamin C supplementation (Group OVC, n=7). After the surgical procedure of ovariectomy, group OVC received 1 g ascorbic acid in 500mL water daily. After 100 days following the ovariectomy, bone mineral density (BMD) was measured by using dual-energy X-ray absorptiometry.
Results: BMD was significantly lower in the group O than in the group C (p=0.015), whereas it was significantly higher in the group OVC than in the group O (p=0.003). MDA activity was significantly higher in the group O than in the group C (p=0.032), whereas it was significantly lower in the group OVC than in the group O (p=0.025). SOD activity was significantly higher in the group O than in the group C (p=0.032). Erythrocyte deformability was significantly higher in the group O than in the group C and OVC (p=0.008, p=0.021, respectively).
Conclusion: Erythrocyte deformability may show negative variations, suggesting a causative role in disruption of blood flow and tissue perfusion, which also negatively affect bone metabolism. Vitamin C supplementation seems to reverse those negative effects of variations in erythrocyte deformability. However, our preliminary results should be confirmed in more experimental studies and clinical trials *(Tab. 3, Ref. 28)*. Full Text in free PDF *www.bmj.sk.*
Key words: osteoporosis, erythrocyte deformability, superoxide dismutase, malondialdehyde.

Osteoporosis (OP) is defined as reduction in total bone mass without any internal deformity, physiologically as a disruption of bone turnover balance toward an increased resorption (1).

It was determined that there is a close relationship between an increased bone turnover and OP formation in the early and late period after the ovariectomy in rats (2). The ovariectomy causes increase in both, bone resorption and production where, because the rate of resorption exceeds the production rate, overall bone mass decreases (3).

Oxidative stress (OS) results from imbalance of free oxygen radicals production and elimination, during anaerobic metabolism (4). Oxidative stress is evident in some pathological conditions like osteoporosis, Respiratory Distress Syndrome, atherosclerosis, chronic renal failure, rheumatoid arthritis, diabetes, sepsis and Alzheimer Diseases (5–10).

Hemorological factors are sensitive to metabolic changes and may be affected by tissue perfusion due to cardiovascular problems. Disorders in the hemoreologic state may lead to an inadequate recovery in plasma viscosity (11). Erythrocyte deformability and plasma viscosity are important factors that affect organ and tissue perfusion (12). For the migration of oxygen and vital molecules to the final organ capillaries and the clearance of metabolic wastes, erythrocytes must be able to extend and curve and have the capability to move in these areas. This capacity is termed as "deformability" (13)

There is an equilibrium in the free radical production and antioxidant defence system that suppress the production of oxygen free radical in the body. Oxidative damage occurs when this equilibrium is disrupted. An increased lipid peroxidation is also one of the result of the increased OS (14). It was shown that some parameters of erythrocyte functions and membrane integrity are impaired in the increased lipid peroxidation in vivo and in vitro studies (15). The products that arise due to lipid peroxidation associated with the increased OS significantly affect the membrane permeability and microviscosity, thus diminishing the deformability capacity and survival of the erythrocytes (16).

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In the study on ovariectomized rats it was stated that malondialdehyde (MDA) levels were markedly increased depending on the oxidative stress induced by ovariectomy (17). In another study it was also shown that erythrocyte catalase (CAT) activity was markedly lower, whereas erythrocyte MDA and nitric oxide (NO) activities were markedly higher in postmenopausal women with respect to the control groups (18).

Vitamin C is an essential factor for both collagen formation and hydroxyproline and hydroxylysine synthesis. A positive correlation between vitamin C and bone mass was shown in epidemiologic studies. Vitamin C is a potent antioxidant by having the ability to reduce the harmful effects of free radicals and laboratory studies demonstrated that vitamin C decreased bone resorption (19, 20).

In this study, we aimed to determine potential changes in antioxidant enzyme levels, lipid peroxodation and erythrocyte deformability and to study the effects of vitamin C supplementation on these changes in ovariectomy induced osteoporotic rats.

Materials and methods

This study was conducted in the Physiology laboratory of Kirikkale University upon the consent of the Experimental Animals Ethics Committee of Kirikkale University.

In the study, 20 female Wistar Albino Rats, 14 weeks aged, grown in the same environment, weighing 175–215 g, were used. Randomized 6 rats were grouped as control and no surgical procedure was performed (Group C, n=6). The rest 14 rats were fed with standard food and water until 2 hours before anesthesia. The rats were kept under 20–21 °C at cycles of 12-hour daylight and 12-hour darkness. Anesthesia was provided by intraperitoneal 50 mg /kg ketamine before surgery. Under the anesthesia, bilateral ovariectomy was performed via laparotomy. Seven rats were randomized as the treatment group among the 14 ovariectomized rats. Vitamin C (L ascorbic acid) was added to the water of the treatment group at the dose of 1 g ascorbic acid in 500 mL water daily. Vitamin C non supported group was named as the ovariectomy group (Group O, n=7), vitamin C supported group was named as the ovariectomy + Vitamin C (group OVC, n=7) group. Twenty ovariectomized rats were kept alive for 100 days to observe the development of OP.

100 days following the ovariectomy, bone mineral density (BMD) was measured. Subsequently, the rats were euthanized to collect blood samples from vessels in abdominal cavity and bone tissue samples from femur. Heparinized total blood samples were used to prepare erythrocyte packs. Deformability measurements were done by erythrocyte suspensions with 55 htc in phosphate buffered saline (PBS) buffer.

BMD measurement

Bone mineral densities of all rats were measured 100 days after ovariectomy prior to euthanasy according to the method of Sobhani et al (21) by using the dual energy X ray absorptiometry (DEXA) (Norland XR-36; Norland Inc., Fort Atkinson, WI, USA) with small subjects' programme (1x1 mm resolution, 60 mm/s sweepspeed). Bone mineral density was determined as the amount of mineral per cubic centimeters of bone (g/cm^2).

Deformability measurements

Erythrocyte deformability was measured using a constant flow filtrometer system (MP 30, Biopac Systems Inc, Commat, USA). Erythrocyte suspension that was delivered at 1ml/min flow rate was passed through a nucleopor-polycarbonate filter of 5µm in diameter, and alterations in the filtration pressure corresponding to different flow rates were measured. The alterations in the pressure were transferred to computer medium with an MP 30 data equation system. The ratio of the values of filtration pressure for the cellular suspension and buffer were calculated, and the relative resistance was calculated.

Measurements of oxidative state parameters

Bone samples were homogenized within the 0.1 M phosphate buffer. Experiments were studied at the upper phase obtained after centrifugation. The SOD (Randox Laboratories Ltd., SD125 Ransod 5x20ml UK) and MDA levels were measured in the bone homogenates. Homogenates protein contents were measured as well and parameters were expressed as values per protein units.

Statistical analysis

The statistical Package for the Social Sciences (SPSS, Chicago, IL, USA) 12.0 program was used for statistical analysis. Variations in bone oxidative state parameters, erythrocyte deformability and bone density between the study groups were assessed by the Kruskal–Wallis test. The Bonferroni adjusted Mann–Whitney U test was used after a significant Kruskal–Wallis to determine which group differs from the other. The results were expressed as the mean ± standart deviation (Mean±SD) and the minimum-maximum (min-max). Statistical significance was set as the p value <0.05 for all analysis and $p<0.033$ (0.1/3) for the Bonferroni adjusted Mann–Whitney U test.

Results

It was demonstrated that BMD levels of the Group O were significantly lower than both the Group C and Group OVC ($p=0.015$, $p=0.003$ respectively) in the evaluation of ovariectomized rats to control whether the ovariectomy provided an appropriate reduction in BMD or not in 100 days after ovariectomy (Tab. 1).

It was determined that ovariectomy caused an increase in the relative resistance of rat erythrocytes that is a marker of erythrocyte deformability ($p<0.05$) (Tab. 2).

The Group C and group OVC erythrocyte deformability indexes were found to be similar ($p>0.05$) whereas the Group O erythrocyte deformability was markedly higher than those of the Group C and the Group OVC ($p=0.008$, $p=0.021$ respectively).

There were significant differences in MDA activities of femur bone tissues among subgroups ($p=0.036$). It was determined that these differences were derived from the difference between

Tab. 1. Bone Mineral Densities of female rats.

	Group C (n=6)		Group O (n=7)		Group OVC (n=7)		P*	P** C-O	P** C-OVC	P** O-OVC
	Mn±SD	min–max	Mn±SD	min–max	Mn±SD	min–max				
Spines (g/cm^2)	0.120±0.007	0.116–0.133	0.113±0.003	0.109–0.118	0.121±0.004	0.114–0.125	0.004	0.015	0.253	0.003

Group C, control group; Group O, ovariectomy group; Group OVC, Ovariectomy + Vitamin C group; Mn±Mean SD – standard deviation
* – significance levels with Kruskal-Wallis test p< 0.05
** – significance levels with Bonferroni corrected Mann-Whitney-U test p< 0.033

Tab. 2. Erythrocyte deformability index values of female rats.

	Group C (n=6)		Group O (n=7)		Group OVC (n=7)		P*	P** C-O	P** C-OVC	P** O-OVC
	Mn±SD	min–max	Mn±SD	min–max	Mn±SD	min–max				
DI	2.63±0.18	2.39–2.86	3.06±0.23	2.78–3.35	2.72±0.18	2.55–3.02	0.015	0.008	0.628	0.021

Group C, control group; Group O, ovariectomy group; Group OVC, Ovariectomy + Vitamin C group; DI, deformability index Mn±Mean SD; standard deviation
* – significance levels with Kruskal-Wallis test p< 0.05
** – significance levels with Bonferroni corrected Mann-Whitney-U test p< 0.033

Tab. 3. Oxidative state parameters of bone tissues of femur in female rats.

	Group C (n=6)		Group O (n=7)		Group OVC (n=7)		P*	P** C-O	P** C-OVC	P** O-OVC
	Mn±SD	min–max	Mn±SD	min–max	Mn±SD	min–max				
MDA (nM/ml)	12.85±6.01	3.58–20.76	24.21±10.21	5.35–35.27	12.63±3.56	8.55–18.58	0.036	0.032	0.775	0.025
SOD (U/ml)	2.28±0.57	1.5–2.78	3.85±1.25	2.44–5.88	2.50±0.44	1.55–2.92	0.049	0.032	0.668	0.048

Group C, control group; Group O, ovariectomy group; Group OVC, Ovariectomy + Vitamin C group; Mn±Mean SD – standard deviation
* – significance levels with Kruskal-Wallis test p<0.05
** – significance levels with Bonferroni corrected Mann-Whitney-U test p<0.033

the Group O and C and between the Group O and the Group OVC. Malondialdehyde activities of the Group O were significantly higher than that of the Group C and OVC (p=0.032, p=0.025 respectively), and the values of the group C and OVC were found to be similar (Tab. 3).

There were differences in SOD activities of femur bone tissue between the groups (p=0.047). Superoxide dismutase activities of the Group O were significantly higher than those of the Group C (p=0.032) (Tab. 3).

Conclusion

Oxygen dependent living organisms have evolved the antioxidant defence system for the protection against the excessive oxygen free radical production. The enzymes like SOD, CAT and GSH-Px constitute the intracellular antioxidant defence mechanisms. These enzymes eliminate the superoxide anions and hydrogen peroxides and inhibit key reactions in the free radical formations (22).

The activities of SOD, which is an antioxidant enzyme, were determined significantly higher in the Group O than the Group K in our study. SOD is the first enzyme in oxygen toxicity defending against the free oxygen radical production, it catalyses the reaction of conversion of O_2 to H_2O_2 (23). The increased SOD activities in ovariectomized rats may be evaluated as a marker of an excessive production of free oxygen radicals.

The increased lipid peroxidation is also observed in increased oxidative stress as a consequence. The products of increased lipid peroxidation may cause an important alteration in erythrocyte membrane permeability and microviscosity. In vitro it was observed that MDA exposure of erythrocyte resulted in a decrease of erythrocyte life span and deformability and it was stated that formation of membrane lipid peroxidation and MDA accumulations due to oxidative stress may play a role in aging of erythrocytes (13, 24).

The measurement of MDA levels that is the end product of lipid degradation, is the most familiar way of determining lipid peroxidation, induced by free oxygen radicals. In our study, a

markedly elevated MDA level in ovariectomized rats was the indicator of an increased lipid peroxidation. These findings are parallel with some studies in literature (18, 25–27). Additionally, we also determined that erythrocyte deformability characteristic of ovariectomized rats were impaired in comparison with the control group and the ovariectomized+vitamin C group. Erythrocyte life span in circulation is known to be affected by factors causing changes in mechanical features, like deformability. Endogenous membrane phospholipid peroxidation is also a part of biological process affecting membrane mechanical actions. In the previous studies it was stated that the exposure of erythrocytes lipids to the agents inducing peroxidation even at low concentration may cause a marked increase of the membrane rigidity, and a marked decrease of its deformability (15, 16).

A positive correlation between the vitamin C and bone mass was shown in epidemiologic studies; low vitamin C intake was associated with an accelerated bone mineral density loss. In one study it was shown that high vitamin C levels had a negative correlation with the fracture formation. However, randomized clinical studies are not available yet (19, 20).

Vitamin C is a potent antioxidant in reducing free radical effects. It was shown that antioxidants limit bone resorption. Other mechanisms of the vitamin C effects on bone mineral density are not clear, on the other hand, it is thought that the vitamin C has a positive role in collagen synthesis during bone matrix formation, in osteoblast maturation or in Calcium absorption (28).

All these data and our results suggested that the increased erythrocyte lipid peroxidation is responsible for the distruption of erythrocyte deformability in OP established rats. Disrupted erythrocyte deformability is the probable reason of various problems seen at microcirculation levels in osteoporotic rats. Probable microcirculation problems may play a role in osteoporosis development via negatively affecting bone metabolisms. It is a subject of another topic to discuss these probable mechanisms. Our results brought to mind that the erythrocyte deformability measurements may benefit in the follow up of patients who are at risk of OP. Additionally, it was shown that, the vitamin C treatment can reverse negative effects of disrupted erythrocyte deformability on bone metabolism. In another words; we think that erythrocyte deformability measurements can give information about negative process in bone metabolism, if it is present; vitamin C supplementation decreases OS in connection with BMD loss.

In this study we suggest that negative changes may be observed on the erythrocyte deformability after ovariectomy, and these negative changes may negatively affects bone metabolisms by resulting in functional impairments on blood flow and tissue perfusion, on the other hand, the vitamin C can reverse the effects. We also have a conviction that these finding should be supported and detailed with clinical and experimental studies having more details and larger series.

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